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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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INVENTOR(S)					
Given Name (first and middle [if any])		Family Name or Surname		Residence (City and either State or Foreign Country)	
NELS JENS		LAURITZEN		PISCATAWAY, NJ	
Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
PURIFIED MARINE COLLAGEN AND PRODUCTS THEREOF					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
<input type="checkbox"/> Customer Number: _____					
OR					
<input checked="" type="checkbox"/> Firm or Individual Name		NELS J. LAURITZEN			
Address		38 ORRIS AVENUE			
Address					
City		PISCATAWAY		State	NJ
Country		MIDDLESEX		Zip	08854
		Telephone	732-699-1533	Fax	732-463-8841
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<input checked="" type="checkbox"/> No.					
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[Page 1 of 2]

Respectfully submitted,

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(if appropriate)

Docket Number:

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# PURIFIED MARINE (Non Mammalian) COLLAGEN And Products Thereof

Specification Document for Provisional Patent Application

Nels J. Lauritzen (11/17/03)

## Purified Marine (Non Mammalian) Collagen

**Background:** Collagen has been an excellent biomaterial with many years of demonstrated biocompatibility and efficacy as a medical device. The majority of medical devices on the worldwide market today employ the use of Type I bovine collagen. Bovine collagen is a risk in that bovine collagen is associated with Bovine Spongiform Encephalitis (BSE). The European Union has put medical device companies on notice that steps need to be taken to insure that the collagen used in their medical devices are free of potential prion and virus transmission. The FDA has taken a position to insure the safety of medical devices containing bovine collagen. Marketers of collagen containing medical devices are ever vigilant to develop test methods and processes to monitor, eliminate and in short, deal with the prion or viral transmission issue. A very strong solution is to source collagen from a specie having no known association with transmissible prions or viral contaminants.

### Opportunities:

The collagen microfibrillar and sponge hemostat as well as the dural regenerative matrix are classified as II Medical Devices by the US FDA and are approvable via 510(k) demonstration of substantial equivalence to a previously marketed device. Product applications employing this Purified Marine Collagen:

- Hemostats

  - Microfibrillar

  - Lyophilized Sponge

- Various Tissue Regenerative Matrices, of which Dural Regenerative Matrix is of particular interest.

- Bio Engineered Products – alone or in combination with others as coatings, films or monofilaments.

### Tissue Source:

The material source for purification processing is marine and has not been known or used as a source previously. Specifically the preferred specie is Tuna and preferred tissues within the Tuna are:

- Caudal Tendon – located in and around the peduncle

- Pectoral Tendon

- Caudal Ray Tendon – lying under the skin and running from muscle tissue to the caudal peduncle.

### **Intercostal Tendons – networked in the ribs**

These robust collagenous tendons have structural and mechanical similarity to tendons found in other vertebrates. Biochemical studies indicate that tuna tendon collagen is composed of the alpha-1/alpha-2 heterotrimer that is typical of vertebrate Type I collagen, while tuna skin collagen has the unusual alpha-1/alpha-2/alpha-3 trimer previously described in other fish skin.

The value of this specific tendon source is in the observation that it can be purified with the result being a collagen structure whose performance is very similar to bovine collagen in both chemical and mechanical characteristics.

### **Specification for Manufacture of Purified Marine Collagen:**

**1.0 Caudal Tendon Preparation** - Slice approximately 1000 grams of frozen tuna caudal tendon using a 'deli' (i.e. NBI Natsune deli slicer). Slice target thickness is 0.012 to 0.15 inches thickness. Weigh the resulting sliced tendon

**2.0 Solids on Sliced Caudal Tendon** - Weigh out  $2.0 \pm 0.5$  gm. (wet weight) sliced tendon into weighing tins and determine solids by drying for 4 hours at 105° C. Three replicate samples are used to insure accuracy.

Initial dry weight of ground caudal tendon should be @ 300 grams (ASSUME @30% SOLIDS). This impacts chemistry mass balance for the remainder of the process.

**3.0 Buffer Preparation** - Prepare 10 liters of 1% NaHCO<sub>3</sub> solution by adding 100 grams of NaHCO<sub>3</sub> to 10 liters of distilled or de-mineralized water. Then add 1N NaOH to the solution to get the pH to 8.5. (1N NaOH is prepared by dissolving 4 grams NaOH in 100ml distilled H<sub>2</sub>O).

\*Note: sequest @300 ml. of the prepared buffer to be used as an enzyme premix in the Enzyme Treatment (immediately following).

**4.0 Enzymatic Treatment** - Add the weighed out sliced caudal tendon to the above solution. Stabilize @ 20°C and add 24 grams of Pancreatin 8X (Sigma) dissolved in 300ml. Of solution taken from previously prepared 10 liter batch.

**5.0 Enzyme Deactivation (Ammonium Nitrate Solution)** - Prepare solution of 10 liters Distilled water, 1000 grams NH<sub>4</sub>NO<sub>3</sub> and 12 grams NaClO<sub>2</sub>. Observe and record pH of solution.

\*Note – Add NaClO<sub>2</sub> to solution during last 5 minutes of preparation/stirring, ideally immediately prior to adding the deactivation solution to the enzyme treatment solution.

Add the Ammonium Nitrate deactivation solution directly to the enzyme treatment to deactivate the enzymatic activity.

Stir intermittently for 1 hour at room temperature (22-25°C).

**Fiber Removal/Transfer** - Separate the caudal tendon fibers from the treatment solutions by straining through a fine mesh screen (@1/32 " open), perforated metal strainer (i.e. China Hat) or Centrifuge the deactivated enzyme treatment solution at 5,000 rpm @15 °C for five (5) minutes.

**6.0 Washing** - Wash three (3) times, 15 minutes for each wash, with 5 liters of distilled water per wash using the centrifuge (5,000 rpm @15°C for 5 minutes) to separate the fibers from the wash water after each washing. Observe and record pH reading of the washes.

\*pH readings should be in the range of 6.0 to 8.0.

**7.0 Alkalai Treatment (Na<sub>2</sub>CO<sub>3</sub> Solution)** - Prepare a solution of 10 liters distilled water, 100 grams Na<sub>2</sub>CO<sub>3</sub>, at pH 11 and 20-25°C.

Place the fibers into the 5 liters of 1% Na<sub>2</sub>CO<sub>3</sub> solution at 20-25°C for 18 hours. Agitate slowly (@70 rpm) using mechanical stirring.

**8.0 Washing** - Wash three (3) times, 5 minutes for each wash, with 3 liters of distilled water (adjusted to pH 8.5 using dilute NaOH) per wash using the centrifuge (5,000 rpm @15°C for 5 minutes) to separate the fibers from the wash water after each washing.

\*Each wash is adjusted to pH 8.35 - 8.5 at the end of each wash cycle. Do not allow the pH to fall below 8.35 otherwise fibers will swell with water and later processing/drying will become very difficult and potentially of lower quality.

**9.0 Solids** - Squeeze out excess water, weigh the wet fiber and run a % solids on the fiber. Three samples are used to determine solids in order to provide accuracy.

**10.0 Lactic Acid Treatment** - Make a 0.7% dispersion using a 0.2% lactic acid (i.e. 2 ml. Lactic acid per 1000 ml. Distilled water). Dispersion batches are made up in 3 liter batches due to current equipment limitations.

Prepare the 0.7% dispersion by adding 21 grams (dry weight) of fiber to 3000 ml. distilled water containing 6 ml. lactic acid.

To determine how much wet weight fiber to use in dispersion making:

Wet Weight = dry weight/% weight solids = 21 grams/% solids = \_\_\_\_\_ gm. wet weight caudal tendon fibers.

Keep the 0.7% dispersion cold (in refrigeration @ 8 - 14° C) for One (1) hour, and allow the fibers to swell.

Waring Blend (3.5 liter Waring Blender) the dispersions three times at 'low', speed for 7 seconds per setting for each batch while keeping the batches at 10 -14° C.

**11.0 Centrifugation** - Using the Sorvall refrigerated centrifuge, centrifuge the dispersion for 5 minutes at 4000 rpm., at 15°C.

Pour off all the supernatant into a clean 25.5 liter vessel (or one of suitable capacity) and the residue into a jar. Do this for each batch, collecting all of the supernatant in the 25.5 quart vessel and all the residue in a jar.

Once all of the supernatant has been collected (possibly requiring two 25.5 liter vessels) re-precipitate the translucent fibers by adding 1N NaOH solution to the centrifuged solution to a

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point where pH 7.0 is reached. To 24 quarts of supernatant approximately 400 ml. 1N NaOH is needed.

PH uniformity is very important. The pH of the initial supernatant is @pH 3. The fibers re-precipitate nicely at pH 7. If the solution is at a lower or higher pH than pH 7, the re-precipitated fibers are not easy to work with (i.e. are sticky and/or swollen).

Centrifuge separate the re-precipitated fibers by centrifuge - 5,000rpm, @ 15°C for 5 minutes. Collect all fibers (sediment) for further processing (washing and drying).

- 12.0 Washing** - Wash the purified fibers three times with 4 liters distilled water to which sufficient dilute NaOH is add to raise the pH to 8.0. Each washing is adjusted to pH 8.0 – 8.35.

\*Note – Centrifuge separate fibers from cleaning water after each wash.

- 13.0 Isopropanol (IPA) Wash** - Place the washed fibers into @ 4 liters (or sufficient IPA to completely cover/soak all fibers) of 100% isopropanol.

Maintain the IPA/fiber mass at 30° C.

Let the fibers remain in the isopropanol for at least two hours with intermittent gentle stirring.

Centrifuge separate the fibers from the IPA wash (5,000rpm, @ 15°C for 5 minutes).

Repeat the process for a second Isopropanol Wash.

- 14.0 Acetone Wash** – Place the washed fibers into sufficient Acetone to cover/soak the fibers completely. Maintain the Acetone/fiber mass at 30° C for one hour with intermittent gentle stirring. Hand squeeze the fibers to express the acetone and repeat a second Acetone Wash.

Hand pluck the fibers and dry at 30°C overnight ( $\geq 8$  hours) or until dry in a through air oven.

- 15.0 Shrink (melt) Temperature:  $T_s$**  - Using the melt-temp apparatus, place a fiber in a glass capillary tube, add some water to the capillary tube to keep the fiber wet. Prepare three samples in the aforementioned method in order to provide adequate accuracy.

Set the variable control knob on setting #4.

Closely watch the fiber as the temperature starts to rise. The melt temperature is considered to be the point at which the fiber collapses.

Do this three times and then take the average.

- 16.0 Trichloroacetic Acid Insolubles (% TCA Insolubles)** - To 100 ml. distilled water add 2.5grams trichloroacetic acid (TCA). Filter this through the glass filter paper using a Buchner funnel and collect the filtered solution. To the filtered 100 ml. 2.5% TCA add 2 grams of dry purified tendon fiber and a magnetic stir-bar in to a 250 ml. beaker.

Place this beaker on a hot plate and bring the temperature up to 90 degrees C. Keep the continuously stirred solution at 90 degrees C for 30 minutes.

After 30 minutes at 90 degrees C, let the solution cool.

At the same time, preheat a glass filter paper at 37 degrees C for 30 minutes. Weigh the filter paper.

Once the solution has cooled down, filter it through the preheated, pre-weighed filter paper using a Buchner funnel.

Wash the filtrate with 500 ml. of distilled water.

Allow the filter paper to dry in the oven (place the filter paper on an aluminum weigh dish) at 40 degrees C for 3 hours. Once the filter paper is dry, reweigh the filter paper:

\* Three samples should be processed in the aforementioned procedure in order to provide appropriate accuracy.

#### 17.0 Comments:

##### Typical Results:

Yield @ 60 %

TCA Insolubles (measure of purity) @ 0.05%

Melt Temperature @ 40 – 50 ° C

##### Documentation:

Lab Book (Nels J. Lauritzen) #100

Batch Records Support Lab Book Entries

Product Application: Materials manufactured by the aforementioned process are intended to be used for...

##### Regenerative Matrices

Dura

Skin

Cartilage

Bone

##### Hemostasis

Microfibrillar

Lyophilized Foam

##### Bio-Engineered Material

Coatings for implanted screws, shafts and stents

Vascular and Neural tube guides

Nels J. Lauritzen

*Nels J. Lauritzen* 11/24/03

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